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Melatonin modulates aromatase activity in MCF-7 human breast cancer cells

Abstract: Most of the current knowledge about the mechanisms by which melatonin inhibits the growth of breast cancer cells point to an interaction of melatonin with estrogen-responsive pathways, thus behaving as an antiestrogenic hormone. However, a possible effect of melatonin on the local synthesis of estrogens had not been examined. The objective of this work was to study whether melatonin may modify the aromatase activity in MCF-7 breast cancer cells thus modulating the local estrogen biosynthesis. In MCF-7 cells cultured with testosterone in estradiol-free media, melatonin (1 nM) counteracts the testosterone-induced cell proliferation dependent on the local biosynthesis of estrogens from testosterone by the aromatase activity of the cells. We found that melatonin reduces the aromatase activity (measured by the tritiated water release assay) of MCF-7 cells both at basal conditions and when aromatase activity was stimulated by cAMP or cortisol. The greatest inhibition of the aromatase activity was obtained with 1 nM melatonin, the same concentration that gives the highest antiproliferative and anti-invasive effects of MCF-7 cells. Finally, by RT-PCR, we found that melatonin downregulates aromatase expression at the transcriptional level in the MCF-7 cells. We conclude that melatonin, at physiological concentrations, decreases aromatase activity and expression in MCF-7 cells. This aromatase inhibitory effect of melatonin, together with its already known antiestrogenic properties interacting with the estrogen-receptor, makes this indoleamine an interesting tool to be considered in the prevention and treatment of hormone-dependent mammary neoplasias.

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Introduction

Melatonin, the main secretory product of the pineal hormone, is an indoleamine secreted during darkness in mammals including humans [1]. This indoleamine acts as a regulator of neoplastic cell growth, particularly on endocrine-responsive breast cancer [see reviews 2–5]. In this concern, the most common conclusion is that melatonin, in vivo, reduces the incidence and growth of chemically induced mammary tumors in rodents [4]. *In vitro*, melatonin, at concentrations corresponding to the physiological levels present in human blood during the night, inhibits proliferation, increases expression of p53 and reduces the invasiveness of the estrogen-responsive MCF-7 human breast cancer cells [4–10]. Although different hypotheses, including the melatonin immunomodulatory actions [11], its antioxidative effects [12], or inhibition of telomerase activity [13] have been postulated to explain the oncostatic properties of melatonin, the effects of this indoleamine on mammary cancer have been mostly considered as a consequence of its antiestrogenic actions. However, the precise mechanisms by which melatonin interferes with estrogen-signaling pathways is only partially understood [5].

Estrogens are the most important endocrine influence identified for the development and mitogenic stimulation of breast cancer [14]. While the ovaries are the principal source of systemic estrogen in the premenopausal nonpregnant woman, other sites of estrogen biosynthesis are present throughout the body and these become the major sources of estrogen after menopause [15]. The biosynthesis of estrogens in peripheral tissues depends on the activity of an enzymatic complex, the cytochrome P450 aromatase and NADPH-cytochrome P450 reductase, which catalyzes the conversion of androgens to estrogens. The high incidence of breast cancer in postmenopausal women suggests that local estrogen synthesis due to the aromatization of androgen in breast tissue plays an important role in the pathogenesis of estrogen-dependent breast cancer [16]. The aromatase activity in breast cancer tissue has been demonstrated to be higher than in nonmalignant breast tissue or tissue distal to tumors, thus leading to the hypothesis that an increased production of estrogens within breast tumors may exert a biological effect and thereby stimulate tumor growth in postmenopausal patients [15, 16]. Therefore, effective inhibition of breast aromatase might be an important modulator of estrogen production in breast cancer cells [17, 18].

In the in vivo and in vitro studies relating melatonin with tumor growth, estradiol reaches the tumor tissue from sources different from the mammary gland tissue (ovaries in in vivo models and estradiol added to the culture medium in in vitro models) [3, 4]. However, there were no studies focusing on the possible effects of this indoleamine on the local synthesis of estrogens. The MCF-7 human breast cancer cells are a good model to study the possible effects of melatonin on the local synthesis of estrogens because it is a well-established estrogen-dependent cell line, in which the cells possess aromatase activity [19] as well as melatonin receptors [20, 21]. The objective of the present work is to test whether melatonin inhibits aromatase activity of MCF-7 cells.

Material and methods

Cells and culture conditions

The MCF-7 human breast cancer cells were purchased from the American Tissue Culture Collection (Rockville, MD, USA). They were maintained as monolayer cultures in 75 cm^2 plastic culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Co., St Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS) (Gibco, Cergy Pontoise, France), penicillin (20 U/mL) and streptomycin (20 μg/mL) (Sigma), at 37°C in a humid atmosphere containing 5% CO_2. Cells were subcultured every 3–4 days by suspension in 5 mM Na_2-EDTA in PBS (pH 7.4) at 37°C for 5 min.

Before each experiment, stock subconfluent monolayers (80%) of MCF-7 cells were incubated with 5 mM Na_2-EDTA in PBS (pH 7.4) at 37°C for 5 min, resuspended in DMEM supplemented with 5% FBS and passed repeatedly through a 25-gauge needle to produce a single cell suspension. Cell number and viability were determined by staining a small volume of cell suspension with 0.4% trypan blue saline solution and examining the cells in a hemocytometer.

Indirect measurement of aromatase activity

Indirect evidence of aromatase activity of estrogen-dependent cells, such as the MCF-7, can be obtained by evaluating cell proliferation in estrogen-free media in the presence of testosterone. Under these conditions, cell growth depends on the biotransformation of androgens to estrogens via the aromatase activity of the cells [22, 23]. To test this, MCF-7 cells were seeded into 96-well culture plates at a density of 8000 cells per well, in DMEM supplemented with 5% FBS, penicillin (20 U/mL) and streptomycin (20 μg/mL), at 37°C in a humid atmosphere containing 5% CO_2. After 48 hr of incubation to allow a correct attachment of the cells, media were changed to ones supplemented with 5% charcoal-stripped FBS (sFBS) containing either testosterone (Sigma-Aldrich Química S.A., Madrid, Spain) (1 μM), melatonin (Sigma-Aldrich) (1 nM), the aromatase inhibitor aminoglutethimide (Sigma-Aldrich) (100 μM), or the diluent of these drugs (ethanol, at final concentration lower than 0.0001% per plate). Cell proliferation was assessed at 3 and 5 days of culture, by using the MTT [3(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method, reading absorbance at 570 nm in a microplate reader. MTT was obtained from Molecular Probes Inc. (Eugene, OR, USA).

In a previous experiment carried out to assess that the proliferation of the MCF-7 cells incubated with testosterone was estrogen dependent rather than androgen dependent, MCF-7 cells were seeded as indicated and incubated in the presence of testosterone (10 μM or 10 nM) in combination with an antiestrogen (1 μM to 10 nM tamoxifen) (Sigma-Aldrich) or an androgen receptor inhibitor (10 μM to 100 nM cyproterone acetate) (Sigma-Aldrich). Cell proliferation was measured after 3 and 5 days of culture.

Direct measurement of cellular aromatase activity

Aromatase activity in MCF-7 cells was measured by the tritiated water release assay, based on the formation of tritiated water during aromatization of a labeled androgenic substrate such as [1β-^{3}H(N)]-androst-4-ene-3,17-dione [24]. MCF-7 cells were seeded onto 60 × 15 mm tissue culture dishes (1.5 × 10^6 cells per dish) in DMEM supplemented with 5% FBS, penicillin (20 U/mL) and streptomycin (20 μg/mL). When a homogeneous monolayer of preconfluent MCF-7 cells was reached on days 2–3 of the experiment, media were aspirated and replaced by fresh media (1 mL per plate) supplemented with 5% sFBS and containing 100 nM [1β-^{3}H(N)]-androst-4-ene-3,17-dione (NEN Life Science Products, Boston, MA, USA) (25–30 Ci/mM) in the presence of melatonin (10 μM, 1 nM or 0.1 pM) or the diluent (ethanol at a final concentration lower than 0.0001%). In other experiments cAMP (100 nM), cortisol (100 nM) and melatonin (1 nM) were combined to assess whether melatonin reversed the aromatase activity induced by cAMP or cortisol. In all cases, at 24 hr of incubation, the culture dishes were placed on ice for 15 min to condense any water vapor and the media were transferred to tubes containing 0.25 ml ice-cold 30% trichloroacetic acid (w/v), vortexed and centrifuged at 1700 g for 20 min. The supernatants were extracted with chloroform, vortexed, set at room temperature for 10 min and then centrifuged at 1700 g for 20 min. The resulting aqueous supernatants were adsorbed with 10% dextran-coated charcoal, vortexed, centrifuged at 1700 g for 20 min and the supernatant added to vials with scintillation cocktail and counted in a beta counter. The amount of radioactivity in water [^{3}H] measured was corrected by subtracting the blank values from each sample, obtained by incubating dishes containing medium with the tritiated androgen but no cells. The values were also corrected by taking into account the fractional retention of tritium in medium water throughout the procedure of incubation and processing, utilizing parallel dishes containing medium plus known amounts of [^{3}H] water (NEN Life Science Products) through incubation and assay. The fractional retention of tritium in medium water throughout the incubation and processing of samples was always higher than 87%.

Measurement of aromatase mRNA

Analysis of the aromatase mRNA was carried out by reverse transcription PCR (RT-PCR) in MCF-7 cells. The total cellular RNA was purified with the Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories Inc., Hercules, CA,

USA) following the manufacturer's instructions. Integrity of RNA was assessed by electrophoresis in ethidium bromide-stained 1.2% agarose-Tris-borate-EDTA gels. The absorbance ratio $A_{260\ \mathrm{nm}}/A_{280\ \mathrm{nm}}$ was greater than 1.8. For cDNA synthesis, 0.5 μg of total RNA were denaturated at 70°C for 10 min and reverse transcribed 50 min at 37°C with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 μL in the presence of 500 ng of oligo (dT)12–18 primer.

The PCR was performed using a set of human aromatase-specific primers [5′-CAAGGTTATTTGATGCATGG (forward primer) and 5′TTCTAAGGCTTTGCGCATGAC (reverse primer)] (Sigma Genosys Ltd, Cambridge, UK). The coding sequence between the two PCR primer sites is interrupted by three introns in the gene. As a control quantification, GADPH mRNA was also carried out by RT-PCR using a set of specific primers [5′CCACCCAGGCAAATTCCATGGCA (forward primer) and 5′-TCTAGACGGCAGGTCAGGTCCACC (reverse primer)].

The PCRs were performed for 34 cycles for semiquantitative analysis using the following temperature profile: 55°C, 45 s (annealing); 72°C, 90 s (extension); and 95°C, 45 s (denaturation). Each product was electrophoresed on ethidium bromide-stained 1.5% agarose-Tris-borate gels.

Statistics

The data on cell proliferation or aromatase activity are expressed as the mean ± S.E.M. Statistical differences between groups were processed by one-way analysis of variance (ANOVA) followed, when appropriate, by the Student–Newman–Keuls test.

Results

Proliferation of MCF-7 cells incubated for 3 or 5 days with sFBS in the presence of 1 μM or 10 nM testosterone was blocked in a dose-dependent manner by the simultaneous administration of tamoxifen and was almost completely abolished by 1 μM of this antiestrogenic drug ($P < 0.001$). On the other hand, the stimulatory effects of testosterone on cell proliferation was not reduced by simultaneous addition of 10 μM or 100 nM cyproterone acetate, an inhibitor of the androgens receptor (Fig. 1; only results at 5 days of culture have been presented). These results suggest that the stimulatory effects of testosterone on cell proliferation are not mediated via androgenic receptors, but via estrogenic receptors.

As expected, testosterone increased proliferation of MCF-7 cells cultured for 5 days in media with sFBS (Fig. 2). This stimulatory effect was reduced ($P < 0.001$) by the aromatase inhibitor aminoglutethimide, thus indicating that, at least in part, cell proliferation was dependent on the formation of estrogens from testosterone by the aromatase activity of the cells. Melatonin (1 nM) was able to counteract the stimulatory effect of testosterone similar to aminoglutethimine, thus suggesting that it also exerts inhibitory effects on aromatase. The aromatase inhibitor effects of aminoglutethimide and melatonin were not additive, as coincubation of the MCF7 cells with both agents counteracted the testosterone-induced cell proliferation to a similar level as to when cells were incubated with the drugs individually (Fig. 2). Melatonin alone has only a weak (not significant) antiproliferative effect, as would be expected due the nature of the culture media in which the concentration of estrogens is almost indetectable.

The aromatase activity of MCF-7 incubated for 24 hr with tritiated androstenedione was estimated by the formation of tritiated water. Fig. 3 shows that melatonin at physiological (1 nM) or pharmacological (10 μM) doses, significantly ($P < 0.001$) decreases aromatase activity of MCF-7 cells. Melatonin also decreased the aromatase activity induced by cAMP (100 nM) or cortisol (100 nM), two well-known inducers of aromatase expression (Fig. 4).

As shown above, melatonin significantly decreases aromatase activity of MCF-7 cells. With the aim of determining whether this inhibitory effect over aromatase activity was due to a downregulation of the aromatase expression at the transcriptional level, we then incubated MCF-7 cells with either 1 nM melatonin or vehicle for 90 min and total RNA was isolated to perform semiquantitative RT-PCR with primers specific for human aromatase. As a control, the same samples were subjected to RT-PCR with primers specific for GADPH. Samples were taken between 27 and 34 cycles, in such a way that a linear relationship between PCR products and amplification cycles was observed. Fig. 5 shows a representative experiment repeated four times with similar results. Melatonin treatment inhibits aromatase mRNA expression in these cells. The densitometric analysis of the chromatographic bands (Fig. 5, bottom) illustrates the magnitude of the downregulation of aromatase expression induced by melatonin.

Discussion

The importance of estrogens in the development of breast cancer is supported by numerous experimental and epidemiological studies [14]. Most hypotheses consider that estrogen binding to ERα or ERβ stimulate cell proliferation, thus increasing the possibility of errors in DNA replication resulting in point mutations [25]. However, direct genotoxic effects of some estradiol metabolites could also explain the estrogen-induced carcinogenesis [26]. At this moment, the two main strategies for the design of anticancer drugs with estrogens as their target are based on either the blockade of the ER (antiestrogens) or the inhibition of estrogen synthesis (aromatase inhibitors) [27].

Ovaries constitute the main site of estrogen synthesis in the premenopausal nonpregnant woman. However, there are other local sources in some tissues, including mammary tissue, which acquires a special importance after menopause [15]. In these tissues, estrogens are produced mainly through aromatization of adrenal androgen precursors (androstenedione and testosterone) and reach tissue concentrations higher than in plasma [16].

Melatonin, the main secretory product of the pineal gland [1], exerts oncostatic effects on breast cancer, as described from in vivo and in vitro studies [2–5]. Much of the current knowledge about the mechanisms by which melatonin inhibits tumor cell growth points to an interaction of melatonin with estrogen-responsive pathways, thus behaving as an antiestrogenic hormone [5, 28–30].

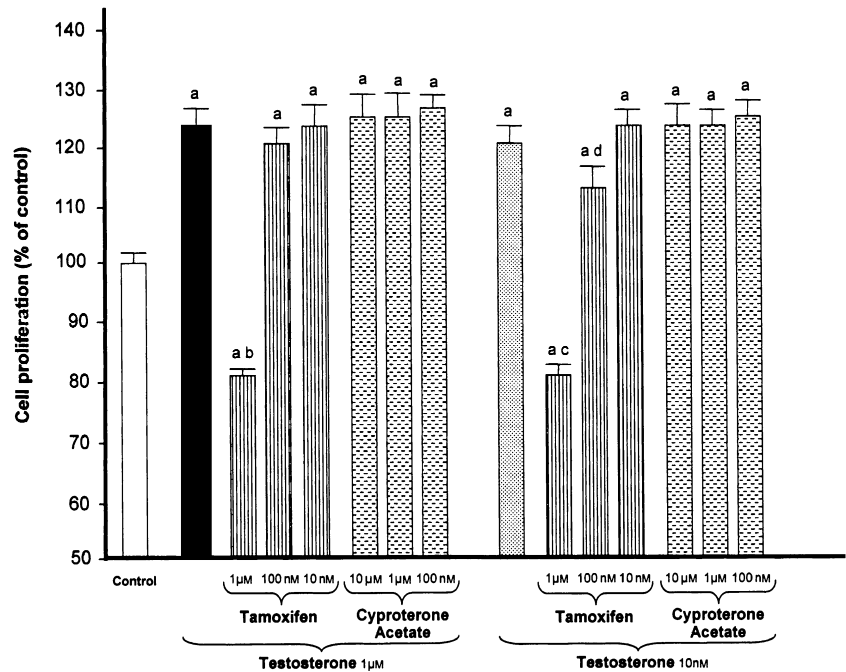

Fig. 1. Effects of tamoxifen (1 μM, 100 and 10 nM) and cyproterone acetate (10, 1 μM and 100 nM) on androgen induced of proliferation of MCF-7 cells. Cells were seeded into 96-well culture plates (8000 cells per well) in medium supplemented with FBS for 48 hr and subsequently for 5 days in medium supplemented with sFBS containing testosterone (1 μM or 10 nM) and the indicated concentrations of tamoxifen or cyproterone acetate. Data are expressed as the percentage of the control group (mean ± S.E.M.). a, $P < 0.001$ versus control; b, $P < 0.001$ versus 1 μM testosterone; c, $P < 0.001$ versus 10 nM testosterone; d, $P < 0.05$ versus 10 nM testosterone.

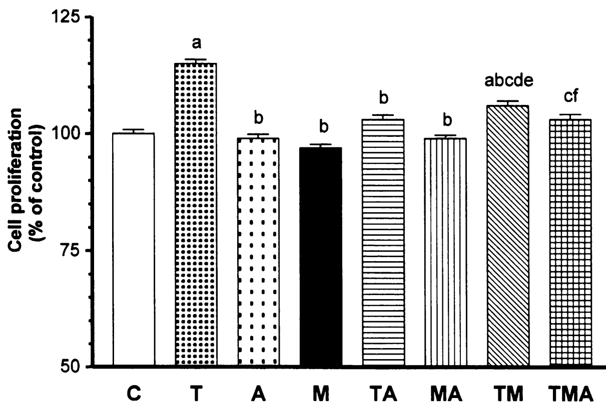

Fig. 2. Effects of 1 μM testosterone (T), 1 nM melatonin (M), 100 μM aminoglutethimide (A), or the diluent of these drugs (ethanol 0.0001%) (C), on MCF-7 cell proliferation. Cells were seeded into 96-well culture plates (8000 cells per well) in medium supplemented with FBS for 48 hr and subsequently for 5 days in medium supplemented with sFBS containing the above-mentioned drugs. Data are expressed as the percentage of the control group (mean ± S.E.M.). a, $P < 0.001$ versus C; b, $P < 0.001$ versus T; c, $P < 0.001$ versus A; d, $P < 0.001$ versus M; e, $P < 0.01$ versus TA; f, $P < 0.01$ versus TM.

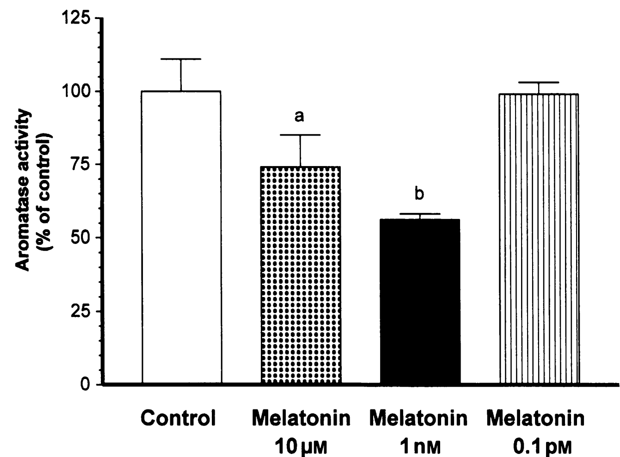

Fig. 3. Effects of melatonin (10 μM, 1 nM or 0.1 pM) or the diluent (ethanol at 0.0001% final concentration) on basal aromatase activity of MCF-7 cells. Cells were seeded onto 60 × 15 mm dishes (1.5×10^6 cells per dish) in DMEM supplemented with 5% FBS for 2–3 days. Then, media were aspirated and replaced by fresh media supplemented with 5% sFBS and containing tritiated androstenedione and the indicated concentrations of melatonin. Aromatase activity was determined after 24 hr of incubation, as described in material and methods. Data are expressed as the percentage of the control group (mean ± S.E.M.). a, $P < 0.05$ versus control; b, $P < 0.001$ versus control.

However, a possible effect of melatonin on the local synthesis of estrogens had not been studied.

The present study demonstrates that melatonin, at physiological (1 nM) and pharmacological (10 μM) concentration, reduces the synthesis of estrogens in MCF-7 cells, through the inhibition of aromatase, the enzyme that catalyzes the rate-limiting step in the conversion of androgens to estrogens [19]. These results are supported by three types of experiments. In the first experimental series, we demonstrate that melatonin counteracts the growth stimulatory effects of testosterone on MCF-7 cells. As testosterone stimulates the proliferation of the MCF-7 cells through its transformation in estrogens which bind to the ER [17, 22, 23], the inhibitory effects of melatonin on the testosterone-induced cell proliferation could be due to the blockade of the formation of estrogens from the androgens.

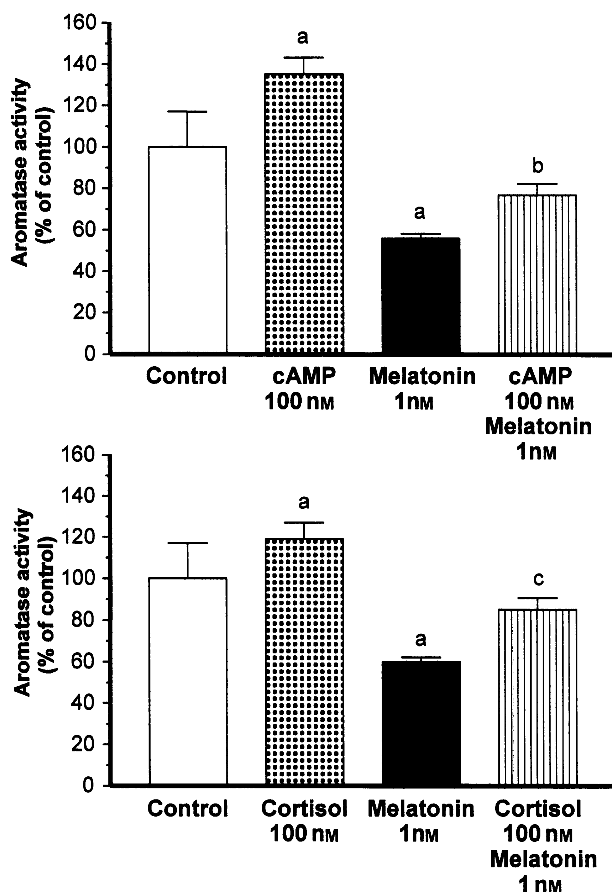

Fig. 4. Effects of 1 nM melatonin on cAMP or cortisol-induced aromatase activity in MCF-7 cells. Cells were seeded onto 60 × 15 mm dishes (1.5×10^6 cells per dish) in FBS supplemented DMEM for 2–3 days and subsequently for 24 hr in medium supplemented with sFBS containing the tritiated androstenedione and cAMP (100 nM) or cortisol (100 nM) in the absence or presence of melatonin (1 nM). Data are expressed as the percentage of the control group (mean ± S.E.M.). a, $P < 0.001$ versus control; b, $P < 0.001$ versus 100 nM cAMP; c, $P < 0.01$ versus 100 nM cortisol.

In a second series of experiments, we directly measured the aromatase activity of MCF-7 cells by quantification of the tritiated water released during the aromatization of a radiolabeled substrate ([1β-^{3}H(N)]-androst-4-ene-3,17-dione) [24]. We found (Figs 3 and 4) that melatonin reduced aromatase activity of MCF-7 cells both under basal conditions as well as when aromatase activity was stimulated by adding cAMP or cortisol [31] to culture media. The greatest inhibition of the aromatase activity of MCF-7 cells was found when melatonin was added at physiological concentrations (1 nM), the same concentration that cause the greatest antiproliferative and anti-invasive effects in these cells [3, 4, 9, 32].

Finally, we evaluated the expression of aromatase at transcriptional level in the MCF-7 cells and its possible modulation by melatonin. For this purpose mRNA was measured by a semiquantitative RT-PCR and, as shown in Fig. 5, a remarkable decrease in mRNA aromatase was induced by incubation with 1 nM melatonin concentration.

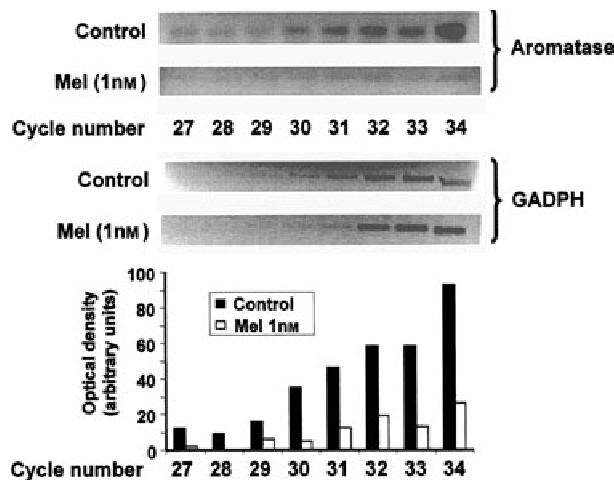

Fig. 5. Effects of melatonin on the expression of aromatase mRNA in MCF-7 cells. Cells were incubated with melatonin (1 nM) or ethanol (0.0001%) (control) for 90 min. Total mRNA was isolated from MCF-7 cells and reverse transcribed. cDNA was subjected to PCR using specific primers for P450 aromatase or GADPH. The results of a representative experiment (chromatogram and densitometric analysis) are shown.

To our knowledge this is the first time a relationship between melatonin and aromatase activity in tumor cells has been clearly established. A limited number of earlier studies had suggested the possible modulatory role of melatonin on the aromatase activity, but mainly related to the function of the neuroendocrine-gonadal axis and with controversial results. Thus, no consistent effect of melatonin on aromatase activity was described in human or bovine granulose cells cultured in a serum-supplemented medium [33]. Other authors attributed the low sperm quality of human seminal plasma to a low aromatase activity dependent on high endogenous melatonin levels [34] or long-term melatonin administration [35]. Finally, a reduction of the aromatase activity in the hypothalamic-preoptic area of adult male Syrian hamsters chronically treated with melatonin, has been described [36] but considered secondary to the decreased circulating levels of testosterone.

Whereas in normal mammary glands the aromatase gene (CYP19) predominantly contains the I.4 promoter, under the tonic control of glucocorticoids, in mammary cancer cells CYP19 genes contain promoters II and I.3 regulated by cAMP [37–39]. Consequently, cAMP activates the transcription of aromatase promoters containing cAMP responsive elements while agents able to decrease cAMP could also decrease aromatase activity. Melatonin, through a membrane-bound Gi protein-coupled MT1 receptor, is reported to downregulate cAMP levels in a variety of cell types [40–42]. In MCF-7 cells, melatonin at concentrations of 10 nM or 1 μM, reduced the forskolin-induced increase of intracellular cAMP [42]. Our group [43] had previously demonstrated that melatonin induced changes in cyclic nucleotide synthesis in murine mammary glands. These changes consisted of a decrease in cAMP and an increase in cGMP accumulation, both responses being dose- and time dependent [43]. Thus, cAMP may be the link between

melatonin and aromatase activity in human breast cancer cells. There is one more possible explanation for the effects of melatonin on aromatase activity. In MCF-7 cells, aromatase activity is stimulated by epidermal growth factor and transforming growth factor-α [31], both of which have been demonstrated to be downregulated by physiological concentrations of melatonin [7, 44, 45]. These findings give rise to the speculation that the mitogenic activity of growth factors may be associated with the modulation of aromatase activity in hormone-dependent human breast cancer cells and melatonin's ability to modulate the synthesis of estrogen-induced growth factors as well as to alter their capacity to act on their cellular targets, may be related to the ability of this indoleamine to modulate aromatase activity and expression.

Therein, we further clarify the well-known properties of melatonin as an antiestrogenic compound able to interact with the ER in breast cancer cells [5] by documenting its ability to reduce the expression of aromatase in these cells and, consequently, to reduce the local synthesis of estrogens. As breast cancer occurs in regions of the mammary gland with the highest levels of aromatase expression [15], the inhibition of aromatase activity and expression by melatonin may be an important mechanism in the ability of this indoleamine to control tumor growth. The coexistence of anti-estrogenic and aromatase-inhibitory properties in the same molecule points to melatonin as an interesting candidate for the treatment and prevention of mammary cancer.

Acknowledgments

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